Bacterial Cyclic \(\beta-(1,2)\)-Glucan Acts in Systemic Suppression of Plant Immune Responses\(^1\)

Luciano Ariel Rigano,\(^a,1\) Caroline Payette,\(^b,1\) Geneviève Brouillard,\(^b\) Maria Rosa Marano,\(^c\) Laura Abramowicz,\(^a\) Pablo Sebastián Torres,\(^a\) Maximina Yun,\(^a\) Atilio Pedro Castagnaro,\(^d\) Mohamed El Oirdi,\(^b\) Vanessa Dufour,\(^b\) Florencia Malamud,\(^a\) John Maxwell Dow,\(^e\) Kamal Bouarab,\(^b,2\) and Adrian Alberto Vojnov\(^b,2\)

\(^a\) Fundación Pablo Cassará, Centro de Ciencia y Tecnología Dr. Cesar Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas, Saladillo 2468 C1440FFX, Ciudad de Buenos Aires, Argentina
\(^b\) Centre de Recherche en Amélioration Végétale, Département de Biologie, Faculté des Sciences, Université de Sherbrooke, Sherbrooke, Québec J1K2R1, Canada
\(^c\) Instituto de Biología Molecular de Rosario, Departamento de Microbiología, Facultad de Ciencias, Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, S2002LRK Rosario, Argentina
\(^d\) Estación Experimental Agroindustrial Obispo Colombres, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Tucumán, T4101XAC Las Talitas, Tucumán, Argentina
\(^e\) BIOMERIT Research Centre, Department of Microbiology, National University of Ireland, Cork, Ireland

Although cyclic glucans have been shown to be important for a number of symbiotic and pathogenic bacterium–plant interactions, their precise roles are unclear. Here, we examined the role of cyclic \(\beta-(1,2)\)-glucan in the virulence of the black rot pathogen \textit{Xanthomonas campestris pv campestris} (Xcc). Disruption of the \textit{Xcc} nodule development \(B\) (ndv\(B\)) gene, which encodes a glycosyltransferase required for cyclic glucan synthesis, generated a mutant that failed to synthesize extracellular cyclic \(\beta-(1,2)\)-glucan and was compromised in virulence in the model plants \textit{Arabidopsis thaliana} and \textit{Nicotiana benthamiana}. Infection of the mutant bacterium in \textit{N. benthamiana} was associated with enhanced callose deposition and earlier expression of the \textit{PATHOGENESIS-RELATED1} (\textit{PR-1}) gene. Application of purified cyclic \(\beta-(1,2)\)-glucan prior to inoculation of the \textit{ndv\(B\)} mutant suppressed the accumulation of callose deposition and the expression of \textit{PR-1} in \textit{N. benthamiana} and restored virulence in both \textit{N. benthamiana} and \textit{Arabidopsis} plants. These effects were seen when cyclic glucan and bacteria were applied either to the same or to different leaves. Cyclic \(\beta-(1,2)\)-glucan–induced systemic suppression was associated with the transport of the molecule throughout the plant. Systemic suppression is a novel counterdefensive strategy that may facilitate pathogen spread in plants and may have important implications for the understanding of plant–pathogen coevolution and for the development of phytoprotection measures.

INTRODUCTION

Plants defend themselves against the majority of microbial pathogens that they encounter through the deployment of both constitutive and induced mechanisms. To be successful, a pathogen must overcome or evade constitutive defenses and suppress or fail to elicit induced defenses (Bushnell and Rowell, 1981; Jackson et al., 1999; Bouarab et al., 2002; Ishiga et al., 2002; Hauck et al., 2003; Abramovitch and Martin, 2004; Keshavarzi et al., 2004; Abramovitch et al., 2006a). In phytopathogenic bacteria, effector proteins delivered by the type III secretion system and the toxin coronatine have been identified as suppressors of induced defenses (Cui et al., 2005; Abramovitch et al., 2006a, 2006b; Janjusevic et al., 2006; Melotto et al., 2006; Nomura et al., 2006). Targets for suppression include the hypersensitive response, expression of defense-related genes, cell wall–based defenses, jasmonic acid signaling, the plant proteosome system, and stomatal closure (Abramovitch et al., 2006a, 2006b; Janjusevic et al., 2006; Nomura et al., 2006).

There appears to be considerable mechanistic diversity in suppression, since different suppressors have different targets. Despite this diversity, a common feature is that, with one exception, all suppressors described to date are reported to be restricted to action in a localized fashion. The exception is the \textit{Pseudomonas syringae} phytotoxin coronatine, which induces systemic susceptibility in \textit{Arabidopsis thaliana}. However, it is unknown whether coronatine itself is systemically transported throughout the plant or whether it acts via local induction of jasmonic acid signaling (Cui et al., 2005). Here, we show that a cyclic glucan of the bacterial phytopathogen \textit{Xanthomonas campestris pv campestris} (Xcc) is systemically transported throughout the plant and can act to suppress particular host defenses both locally and systemically. Our findings thus extend the concept of the systemic suppression of plant defenses, a
phenomenon that may impinge on the spread of bacterial disease in plants.

Xcc is a major bacterial pathogen of cruciferous plants worldwide (Williams, 1980). The ability of Xcc to incite disease in plants depends on the rpf (for regulation of pathogenicity factors) gene cluster (Williams, 1980; Barber et al., 1997; Dow and Daniels, 2000). Several rpf genes are involved in the positive regulation of the synthesis of extracellular factors such as degradative enzymes, cyclic glucan, and the exopolysaccharide xanthan (Slater et al., 2000; Vojnov et al., 2001). Xanthan and certain extracellular enzymes are established as key virulence factors for Xcc (Williams, 1980; Newman et al., 1994; Denny, 1995; Slater et al., 2000; Chang et al., 2001; Vojnov et al., 2001) and it was suggested recently that xanthan has multiple roles during disease, including the suppression of local plant defense (Yun et al., 2006).

Xcc produces an extracellular cyclic glucan that contains 16 glucosyl residues with 15 β-1,2 linkages and 1 α-1,6 linkage (York, 1995). Cyclic glucans are produced by a number of bacteria, including Xcc, Ralstonia solanacearum, Agrobacterium tumefaciens, Bradyrhizobium japonicum, Rhizobium melliloti, and Brucella abortus (Bhagwat et al., 1993, 1999; Breedveld and Miller, 1994; Castro et al., 1996; Dunlap et al., 1996; Talaga et al., 1996; Ingram-Smith and Miller, 1998; Vojnov et al., 2001; Arellano-Reynoso et al., 2005). A glucosyltransferase encoded by the ndvB (for nodule development B) gene has been shown to be required for cyclic glucan biosynthesis in the Rhizobiaceae (Ielpi et al., 1990; Bhagwat et al., 1992; Castro et al., 1996; Iñon de Iannino et al., 1998). Cyclic glucans have been shown to be important for a number of symbiotic and pathogenic plant–microbe interactions (Breedveld and Miller, 1994; Mithofer et al., 2001). They are required for effective nodule invasion in symbiotic nitrogen-fixing R. melliloti and for crown gall tumor induction in A. tumefaciens (Breedveld and Miller, 1994).

The precise roles of cyclic glucans during plant–bacterium interactions are not clear. It has been suggested that a cyclic glucan from B. japonicum promotes symbiosis by the suppression of defense responses, where it may act to antagonize the binding of microbial elicitors of plant defenses (Bhagwat et al., 1993; Mithofer, 2002). It was shown recently that pathogenic Brucella uses the cyclic β-(1,2)-glucan to avoid animal and human innate immune responses (Ugale, 1999; Arellano-Reynoso et al., 2005). These observations prompted us to examine the role of Xcc cyclic glucan in the suppression of plant defenses and the promotion of bacterial pathogenesis.

RESULTS

Cyclic β-(1,2)-Glucan Is an Xcc Factor That Is Essential for Virulence

To test the role of cyclic glucan in Xcc pathogenesis, we generated a null mutant (ndvB−) by disruption of XC_4168, a homolog of the ndvB gene that has an established role in cyclic glucan synthesis in other bacteria (Bhagwat et al., 1993; Castro et al., 1996; Mah et al., 2003). Insertional inactivation of ndvB (Figure 1A) compromised the production of the extracellular cyclic β-(1,2)-glucan (Figure 1B) but had no effect on bacterial growth or on the production of the exopolysaccharide xanthan (data not shown).

Effects of the inactivation of ndvB on disease were first investigated in the model host plant Nicotiana benthamiana. Symptoms and bacterial growth in plants were checked after inoculating the leaves with a 107 colony-forming units (cfu)/mL suspension of Xcc–wild type, Xcc–ndvB+, or Xcc–ndvB− (ndvB− complemented with the Xcc ndvB gene). Five days after inoculation, Xcc strain 8004 (wild type) gave disease symptoms in N. benthamiana leaves (Figure 1C). To assess bacterial growth, leaf discs were bored from the infiltrated area, ground in 10 mM MgCl2, and serially diluted to measure bacterial numbers (see Methods). Bacterial numbers in the zone of infiltration increased by nearly 3 orders of magnitude over 3 d for strain 8004 (Figure 1D). The growth of the wild-type Xcc strain in N. benthamiana is thus very similar to that in the susceptible host turnip (Brassica rapa cv Just Right) (Newman et al., 1994; Yun et al., 2006). By contrast, the ndvB− strain showed only a slight chlorosis with limited necrosis after 5 d (Figure 1C) and had 20-fold lower final population after the same period (Figure 1D). The production of cyclic glucan and the disease were fully restored by complementation of the ndvB− strain with the Xcc ndvB gene (ndvB+ strain) (Figures 1B to 1D). This results suggest that cyclic β-(1,2)-glucan is essential for Xcc virulence on N. benthamiana.

We then investigated whether the growth of Xcc in N. benthamiana was accompanied by induction of the gene encoding the PATHOGENESIS-RELATED1 (PR-1) protein. RNA gel blot analysis was performed with RNA extracted from N. benthamiana leaves at 6, 12, 24, and 48 h after infiltration with a 107 cfu/mL suspension of Xcc 8004, ndvB−, or ndvB+ (see Methods). In response to Xcc strain 8004, the accumulation of PR-1 transcripts was barely detectable at 12 h and reached a maximum accumulation at 24 h after inoculation (Figure 1E). By contrast, with the ndvB− strain, accumulation of PR-1 transcripts reached high levels at 12 h that were maintained until 24 h after inoculation. No PR-1 transcripts were detected in mock-inoculated plants (data not shown). These data suggest that N. benthamiana exhibits a resistance response to the ndvB− strain that includes PR-1 expression.

Several laboratories have demonstrated that callose, a β-(1,3)-glucan with (1,6) modifications, is required for disease resistance against some pathogens (Stone and Clarke, 1992; Hamiduzzaman et al., 2005). We recently showed that enhanced synthesis of this polymer is associated with increased resistance of N. benthamiana to strains of Xcc (Yun et al., 2006). Consequently, we tested whether callose synthesis is associated with the reduction of growth of the ndvB mutant in planta. N. benthamiana leaves were inoculated with 107 cfu/mL suspensions of Xcc strains 8004, ndvB−, ndvB−, and callose deposition was monitored at 48 h after infection by staining the inoculated leaves with aniline blue. Cytological observations were performed at the sites of infection using ultraviolet fluorescence microscopy. Leaves challenged with the Xcc ndvB− strain showed considerably enhanced staining compared with leaves inoculated with the wild-type strain or ndvB+ (Figure 2). The absence of disease symptoms, together with the lower bacterial numbers attained, the more rapid induction of PR-1, and alteration in the plant cell wall, suggest that the host is exhibiting a resistance response to the ndvB− strain.
Figure 1. Infection of *N. benthamiana* with Xcc Strains.

(A) A 1.3-kb fragment of chromosomal DNA containing a 5' portion of the ndvB gene and the 3' end of the ynaJ gene was amplified by PCR using Xcc chromosomal DNA as a template and cloned in pGEM T-Easy (Promega). The 2-kb fragment containing the Spc' cassette from pHP45 (Fellay et al., 1989) was ligated as a SmaI fragment into pGEM T-Easy digested with SmaI within the ndvB gene. The resulting ndvB::Spc' allele was cloned as a 3.3-kb NotI fragment into the sacB suicide vector pJQ200 KS digested with NotI.

(B) DNA gel blot (left) and TLC (right) analysis of Xcc strains for cyclic glucan production. Genomic DNA was prepared from the wild-type strain 8004 and the ndvB' mutant. Genomic DNA was digested with SacI and separated on a 0.8% agarose gel. The blot was probed with the 0.6-kb DNA fragment.

(C) DNA gel blot (left) and TLC (right) analysis of Xcc strains for cyclic glucan production. Genomic DNA was prepared from the wild-type strain 8004 and the ndvB' mutant. Genomic DNA was digested with SacI and separated on a 0.8% agarose gel. The blot was probed with the 0.6-kb DNA fragment.

(D) Bacterial growth (Log cfu/cm²) over time for different strains. The graph shows the growth pattern for 8004, ndvB', and ndvB' mutant strains over 5 days after inoculation.

(E) The figure shows an analysis of PR1 expression over time. The expression levels are normalized to rRNA.
Xcc Cyclic β-(1,2)-Glucan Induces Host Susceptibility through the Suppression of Plant Defenses

To investigate the proposed role of the Xcc extracellular cyclic β-(1,2)-glucan in the suppression of local plant defense, *N. benthamiana* leaves were preinfiltrated with purified glucan (50 μg/mL) at 24 h before inoculation with a 10^7 cfu/mL suspension of Xcc strains. Interestingly, leaves preinfiltrated with cyclic β-(1,2)-glucan now showed disease symptoms in response to the Xcc ndv-B strain (Figure 3B), whereas leaves that had been preinfiltrated with water as a control did not (Figure 3A). Bacterial growth in the zone of infiltration was assessed at 3 d after inoculation. The number of bacteria recovered from leaves that had been pretreated with purified cyclic β-(1,2)-glucan was ~12-fold higher than the number recovered from leaves pretreated with water (Figure 3C). Pretreatment with cyclic β-(1,2)-glucan also enhanced the susceptibility of *N. benthamiana* leaves to wild-type strain 8004; thus, disease symptoms appeared earlier on leaves pretreated with cyclic β-(1,2)-glucan in comparison with water-treated leaves (data not shown). The number of wild-type bacteria recovered at 3 d after inoculation from leaves that had been pretreated with purified cyclic β-(1,2)-glucan was ~9-fold higher than the number recovered from leaves pretreated with water (Figure 3E). Transcript levels of PR-1 were detected by RNA gel blot analysis of total RNA extracted from ndv-B-infected *N. benthamiana* leaves pretreated with water or cyclic β-(1,2)-glucan. A clear reduction of PR-1 expression was observed in leaves pretreated with cyclic β-(1,2)-glucan compared with those pretreated with water (Figure 3D).

Callose deposition was then assessed in *N. benthamiana* leaves that had been pretreated with either water or cyclic β-(1,2)-glucan (50 μg/mL) at 24 h before inoculation of Xcc strains. Callose staining was performed at 48 h after infection. Leaves pretreated with cyclic β-(1,2)-glucan showed reduced callose synthesis when challenged with the Xcc ndv-B strain compared with leaves pretreated with water (Figures 2C and 2E). Callose deposition was also compromised in leaves challenged by the Xcc ndv-B strain (Figures 2D and 2E). From these data, we concluded that cyclic β-(1,2)-glucan permits Xcc ndv-B to grow in its host and allows the establishment of disease in *N. benthamiana* through the suppression of plant defenses that include PR-1 expression and cell wall alterations. The glucan effect was dose-dependent and required a period of time between inoculation of glucan and inoculation of the ndv-B strain to be effective; the minimum concentration of cyclic glucan required for local suppression of defense responses was 10 μg/mL (Figure 4), and the minimal time between glucan treatment (50 μg/mL) and bacterial inoculation for the suppression effect to be observed was 6 h (Figure 5). We estimate that the level of cyclic glucan produced by a 48-h culture in minimal medium of the Xcc wild-type strain 8004 was 30 μg/mL (data not shown). This suggests that the concentration of cyclic β-(1,2)-glucan used in this study was physiologically relevant.

Xcc Cyclic β-(1,2)-Glucan Acts as a Systemic Signal to Suppress Plant Defense

We extended our experiments to ask whether the effects on the suppression of resistance responses could be induced systemically. Leaves of *N. benthamiana* were preinoculated with a 10^7 cfu/mL suspension of wild-type strain 8004, and 24 h later nontreated leaves were inoculated with a 10^7 cfu/mL suspension of Xcc ndv-B strain. Bacterial numbers were assessed at 3 d after inoculation (Figure 6). Remarkably, the later challenged leaves showed disease symptoms in response to the mutant. The number of ndv-B bacteria recovered was approximately ninefold higher than the number recovered from a control in which leaves were preinoculated with ndv-B bacteria (Figure 6). This suggested that the wild-type strain, but not the ndv-B mutant, could suppress disease resistance in *N. benthamiana* in a systemic fashion. To examine the role of cyclic β-(1,2)-glucan in these systemic effects, leaves of *N. benthamiana* were preinfiltrated with water or purified Xcc cyclic β-(1,2)-glucan (50 μg/mL), and 24 h later both preinfiltrated and nontreated leaves were inoculated with a 10^7 cfu/mL suspension of Xcc ndv-B strain. The results (Figure 7) showed that Xcc cyclic β-(1,2)-glucan also suppressed disease resistance in *N. benthamiana* in a systemic fashion. Leaves preinfiltrated with Xcc cyclic β-(1,2)-glucan and challenged with the ndv-B strain showed normal disease symptoms (Figure 7C), as described above. However, leaves that were not inoculated directly with Xcc cyclic β-(1,2)-glucan also showed disease after challenge by the ndv-B strain (Figure 7D). These effects were not observed in water-treated plants (Figures 7A and 7B). Transcript levels of PR-1 were assayed in leaves of *N. benthamiana* that were preinfiltrated with water or purified Xcc cyclic β-(1,2)-glucan and then inoculated with the Xcc ndv-B strain, both in preinfiltrated and nontreated leaves. RNA gel blot analysis of total RNA showed that Xcc cyclic β-(1,2)-glucan also suppressed PR-1 gene expression in a systemic fashion (Figure 7E). The systemic glucan effect required a minimum of 30 μg/mL glucan (Figure 4) and a minimum period of 12 h between inoculation of glucan (50 μg/mL) and inoculation of the ndv-B strain (Figure 5).

As shown in Figure 2, one of the local targets of Xcc cyclic β-(1,2)-glucan is the suppression of callose deposition. Therefore, we tested whether cyclic β-(1,2)-glucan could act as a
systemic suppressor of callose deposition. Leaves of *N. benthamiana* were preinfiltrated with water or purified Xcc cyclic \(\beta-(1,2)\)-glucan (50 \(\mu\)g/mL), and 24 h later nontreated leaves were inoculated with a 10^7 cfu/mL suspension of the *ndaB* mutant. Callose deposition was monitored by staining the infected leaves with aniline blue at 48 h after infection. Leaves from plants pretreated with water showed a considerably enhanced staining upon challenge by the *ndaB* strain compared with leaves from plants pretreated with cyclic \(\beta-(1,2)\)-glucan (see Supplemental Figure 1 online).

We then investigated whether the systemic effects of Xcc cyclic \(\beta-(1,2)\)-glucan on defense suppression were associated with the systemic spread of the molecule itself. Radiolabeled cyclic \(\beta-(1,2)\)-glucan was generated by incubation of a total

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**Figure 2.** Callose Deposition in *N. benthamiana* Leaves Is Associated with Resistance and Is Suppressed by the Xcc Extracellular Cyclic \(\beta-(1,2)\)-Glucan.

(A) to (D) *N. benthamiana* leaves after inoculation with strains of Xcc stained for callose deposits (white dots) observed by fluorescence microscopy: Xcc strain 8004 (A); Xcc *ndaB* strain (B); Xcc *ndaB* strain inoculated after cyclic \(\beta-(1,2)\)glucan pretreatments (C); and Xcc *ndaB* strain (D). Bars = 200 \(\mu\)m.

(E) Average numbers of callose deposits per field of view (0.45 mm²). Error bars represent SD values from three leaves of each plant and three independent experiments. Differences between the response to the *ndaB* mutant after water pretreatment and all other treatments were significant as assessed by Student’s t test at \(P < 0.001\). dpi, days after inoculation.

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**Figure 3.** Xcc Extracellular Cyclic \(\beta-(1,2)\)-Glucan Compromises the Disease Resistance in *N. benthamiana*.

(A) and (B) Disease symptoms on *N. benthamiana* leaves preinfiltrated with water (A) or purified cyclic \(\beta-(1,2)\)-glucan (50 \(\mu\)g/mL) (B) and subsequently infected with a 10^7 cfu/mL suspension of the Xcc *ndaB* strain.

(C) Effects of cyclic glucan and water (control) pretreatments on Xcc *ndaB* strain growth. The mean and SD of three separate measurements of bacterial numbers are given for each time point.

(D) Effects of cyclic glucan and water (control) pretreatments on PR-1 gene expression induced by the Xcc *ndaB* strain. Transcript levels were analyzed at 24 h after bacterial inoculation by RNA gel blot.

(E) Effects of cyclic glucan and water (control) pretreatments on Xcc wild-type (strain 8004) growth. Bacterial numbers were measured immediately after bacterial inoculation and at 3 d after inoculation. Error bars represent SD values. Data sets marked with asterisks are significantly different from control (water-pretreated leaves) as assessed by Student’s t test (\(P < 0.001\)).
membrane preparation from Xcc 8004 with UDP-\([^{14}C]\)-D-glucose (see Methods). Leaves from 4-week-old plants were infiltrated with \([^{14}C]\)cyclic \(\beta\)-(1,2)-glucan, and the radioactivity in both infiltrated and distant leaves was assessed at different time points. As shown in Figure 7F, the radioactivity per unit of area decreased with time in leaves infiltrated with \([^{14}C]\)cyclic \(\beta\)-(1,2)-glucan, whereas distant leaves showed a significant increase in radioactivity at 6 h after inoculation. To test whether the observed radioactivity corresponded to \([^{14}C]\)cyclic \(\beta\)-(1,2)-glucan and not to potential degradation products, we performed gel exclusion chromatography on extracts from distant leaves.

The elution profile of this material (Figure 7G) was identical to that of the authentic (unlabeled) cyclic \(\beta\)-(1,2)-glucan (data not shown) and the \([^{14}C]\)cyclic \(\beta\)-(1,2)-glucan originally applied to the leaves (Figure 7G). In addition, the \([^{14}C]\)-labeled material extracted from distant leaves of \(N.\) benthamiana had the same behavior on thin-layer chromatography (TLC) as the authentic extracellular \([^{14}C]\)cyclic \(\beta\)-(1,2)-glucan, and both the plant-extracted and the authentic materials showed the same pattern of partial and total

**Figure 4.** The Xcc Cyclic \(\beta\)-(1,2)-Glucan Suppression Effect is Dose-Dependent.

Leaves of 4-week-old plants were preinfiltrated with either different concentrations of Xcc cyclic \(\beta\)-(1,2)-glucan or water at 24 h before either the same (A) or distant (B) leaves were inoculated with a \(10^7\) cfu/mL suspension of the Xcc \(ndvB\) mutant strain. Numbers of mutant bacteria were assessed immediately upon inoculation and 3 d later. The mean and SD of three separate measurements of bacterial numbers are given. Data sets marked with asterisks are significantly different from control (water-pretreated leaves) as assessed by Student’s \(t\) test at \(P < 0.001\).

**Figure 5.** Time Dependence for Cyclic \(\beta\)-(1,2)-Glucan to Establish Susceptibility to the Xcc \(ndvB\) Mutant.

Leaves of 4-week-old plants were preinfiltrated with either Xcc cyclic \(\beta\)-(1,2)-glucan (50 \(\mu\)g/mL) or water, and either the same (A) or distant (B) leaves were inoculated with a \(10^7\) cfu/mL suspension of the Xcc \(ndvB\) mutant strain at different times after infiltration. Numbers of mutant bacteria were assessed immediately upon inoculation and 3 d later. The mean and SD of three separate measurements of bacterial numbers are given. Data sets marked with asterisks are significantly different from control (water-pretreated leaves) as assessed by Student’s \(t\) test (\(P < 0.001\)).
acid degradation products as resolved by TLC (Figure 7H). Interestingly, the 14C-labeled material extracted from distant leaves of N. benthamiana was also insensitive to the action of Gluusulase (data not shown). Gluusulase is an enzyme preparation that preferentially degrades linear rather than cyclic glucans (Zorreguieta et al., 1985). Together, these properties suggested that the radiolabeled product recovered from distant leaves was cyclic β-(1,2)-glucan. By extension, our findings suggest that the systemic effects of cyclic β-(1,2)-glucan on the suppression of plant defense could be due to the systemic spread of the molecule itself. However, we cannot exclude the possibility that the local interaction between cyclic glucan and the host generates another suppressing compound that moves systematically.

Cyclic Glucan Suppression Also Occurs in Arabidopsis

We wished to test whether the cyclic β-(1,2)-glucan suppression phenomenon could also be observed in Arabidopsis, a member of the Brassicaceae family that contains the natural hosts for Xcc. Four-week-old Arabidopsis Columbia (Col-0) plants were inoculated by dipping in bacterial suspensions of the Xcc strains 8004 (wild type), ndvB–, and ndvB+ containing 107 cfu/mL in 10 mM MgCl2 and 0.02% Silwet L-77. Control plants were dipped in a solution of 10 mM MgCl2 and 0.02% Silwet L-77. After dipping, plants were covered with a transparent lid and disease symptoms were then observed at 6 d after inoculation. As shown in Figure 8, Xcc strain 8004 (wild type) gave disease symptoms in Arabidopsis leaves after 6 d (Figure 8A). By contrast, no disease symptoms were visible in Col-0 plants inoculated with the ndvB– strain at the same time point (Figure 8A). Bacterial growth within Arabidopsis Col-0 was consistent with these visible differences in disease symptoms. Thus, populations of the ndvB– strain were >100-fold lower than those of the wild-type strain 8004 at 3 d after dip-inoculation (Figure 8B). The ability to cause disease was fully restored by complementation of the ndvB– strain with the Xcc ndvB gene (ndvB+ strain; Figures 8A and 8B).

We then investigated whether the Xcc cyclic β-(1,2)-glucan could promote the virulence of Xcc strains in Arabidopsis. A solution of Xcc cyclic β-(1,2)-glucan (50 μg/mL) was infiltrated into 4-week-old Arabidopsis Col-0 plants at 24 h before dip inoculation with a 107 cfu/mL suspension of the ndvB mutant strain. Symptoms were assessed at 6 d after bacterial inoculation. Plants infiltrated with water were used as a control. Interestingly, plants infiltrated with cyclic β-(1,2)-glucan now showed disease symptoms in response to the Xcc ndvB– strain (Figure 8C), whereas plants that had been infiltrated with water did not (Figure 8C). Bacterial growth was assessed at 3 d after inoculation. The number of bacteria recovered from leaves that had been infiltrated with purified cyclic β-(1,2)-glucan was ~50-fold higher than the number recovered from plants infiltrated with water (Figure 8D).

Systemic effects of Xcc cyclic β-(1,2)-glucan such as those observed in N. benthamiana were also examined in Arabidopsis. For these experiments, a solution of cyclic β-(1,2)-glucan (50 μg/mL) was infiltrated into one leaf of 4-week-old Arabidopsis Col-0 plants, and 24 h later the entire plant was dip-inoculated with a suspension of the ndvB mutant at 107 cfu/mL. Initial infiltration with water was used as a control. Leaves infiltrated with Xcc cyclic β-(1,2)-glucan and then challenged with the ndvB– strain showed disease symptoms (Figure 8E), as described above. Remarkably, leaves that were not directly infiltrated with the Xcc cyclic β-(1,2)-glucan also showed disease after challenge with the ndvB– strain (Figure 8E). Control leaves did not show any visible symptoms (Figure 8E). These results suggested that cyclic β-(1,2)-glucan induces both local and systemic susceptibility to Xcc in Arabidopsis.

DISCUSSION

The intimate relationship between plants and phytopathogens has led to the coevolution of a number of complex strategies for attack and defense. For a pathogen to colonize a host successfully,
it must develop mechanisms either to evade detection or, failing that, to subvert the defense responses (Ritter and Dangl, 1996; Jamir et al., 2004; Metz et al., 2005; Nomura et al., 2005). We have shown here that the cyclic β-(1,2)-glucan of Xcc is a factor that can contribute to the ability of Xcc to cause disease through the suppression of plant defenses associated with PR gene induction and host cell wall modification. This pattern of activity is similar to that of the Pseudomonas syringae type III effector AvrPto, which downregulates the expression of a set of Arabidopsis genes encoding both putatively secreted cell wall and defense proteins in a salicylic acid–independent manner (Hauck et al., 2003) and differs from other effectors such as AvrPtoB, which induce plant disease susceptibility in N. benthamiana and other plants by inhibition of host hypersensitive response–related
programmed cell death (Abramovitch et al., 2003, 2006a; Abramovitch and Martin, 2004).

Our results suggest that cyclic β-(1,2)-glucan generated by Xcc bacteria colonizing one leaf can be translocated to other leaves to induce susceptibility to Xcc, thus promoting bacterial spread through the plant. The ability of the cyclic β-(1,2)-glucan of Xcc to act as a systemic effector of the suppression of host defense responses distinguishes it from the action of almost all suppressors described to date, which as far as we are aware have only been reported to act locally. The only suppressor
shown to induce systemic susceptibility is coronatine; however, it is still unknown whether this toxin acts as a systemically translocated signal or exerts its effect via local activation of the jasmonic acid pathway (Cui et al., 2005). On the other hand, some bacterial toxins have been shown to be able to spread in plants in the absence of the producing pathogens (Mitchell and Bielecki, 1977), supporting our finding that cyclic β-(1,2)-glucan is able to move in host plants in the absence of Xcc.

The local resistance induced by avirulent pathogens around the plant infection site is typically accompanied by the establishment of a systemic immunity to subsequent infection by a range of normally virulent pathogens, the so-called systemic acquired resistance (SAR) (Dangl and Jones, 2001). The nature of the mobile signal responsible for establishing SAR remains unclear. Salicylic acid participates in the local and systemic response, but SAR does not require long-distance translocation of salicylic acid (Vernooij et al., 1994; Ryals et al., 1995). It was recently shown that jasmonates are central to systemic defense, possibly acting as the initiating signal for SAR (Truman et al., 2007). The establishment of a role in systemic signaling for SFD1 (for suppressor of fatty acid desaturase; Nandi et al., 2004) and DIR1 (for defective in induced resistance), encoding a putative lipid-transfer protein (Maldonado et al., 2002), implicates lipids in this phenomenon, but the identity of the transmitted molecule(s) remains elusive (Grant and Lamb, 2006). It will be interesting to determine whether SFD1 or DIR1 is one of the targets of Xcc cyclic glucan action. The kinase-like systemic susceptibility induced by cyclic glucan appear to be similar to those of the systemic activation of wound-responsive proteinase inhibitor (PI) genes in tomato (Solanum lycopersicum) plants (Li et al., 2002), which depend on jasmonate signaling. Thus, the systemic expression of PI genes requires a minimum of 8 h after wounding treatment (Li et al., 2002), and in our experiments a minimum of 6 and 12 h are necessary for Xcc cyclic glucan to suppress plant disease resistance in local and systemic fashion, respectively.

Systemic suppressive effects may have a profound impact on the severity of plant disease, affecting the spread of lesions in the initially challenged leaf and promoting the ability of the pathogen to spread throughout the plant. Xcc is capable of systemic spread through the vascular system of host plants (Williams, 1980), which supports the systemic action of the cyclic β-(1,2)-glucan suppressor. The ability of pathogens to establish systemic susceptibility could act as a counter to the ability of plants to mount systemic defenses (Van Loon et al., 1998; Durrant and Dong, 2004). It will be of interest to examine the interplay between the cyclic β-(1,2)-glucan–induced effects we report here and both the systemic defense responses of SAR (Durrant and Dong, 2004) and the induced systemic resistance, which can be triggered by the association of beneficial bacteria with the roots (Van Loon et al., 1998).

In summary, the results presented here highlight a new strategy used by Xcc to compromise local and systemic plant immune responses in both N. benthamiana and Arabidopsis. These data present a conceptual stride forward in understanding the role of suppression in plant disease. An exciting future challenge will be the biochemical and genetic elucidation of this systemic susceptibility mechanism in N. benthamiana and Arabidopsis, which may have implications for the development of phytotoprotection measures. A second key challenge will be to examine the role of systemic suppression in plant–pathogen coevolution by establishing whether other pathogens employ similar mechanisms to promote the colonization of plants as well as other eukaryotic hosts.

METHODS

Bacterial Strains and Culture Conditions

Xanthomonas campestris pv. campestris (Xcc) strain 8004 (wild type) was described previously (Newman et al., 1994; Vojnov et al., 2001). The glucan-minus mutant was created by disruption of the ndvB gene homolog in Xcc 8004. Strains in which the ndvB gene was disrupted were created with the use of the plasmid pJQ200KS. A 1.3-kb fragment of the ndvB gene (Xc_4188) was amplified by PCR using Xcc chromosomal DNA as a template and the primers 5′-Xcc 8004 ndvB (5′-CAATCTCCG- CATCGTCTCTGG-3′) and 3′-Xcc 8004 ndvB (5′-GTCGGCGGGGATGAGAA- GAACA-3′) and cloned into pGEM T-Easy vector according to the manufacturer’s instructions (Promega). The identity of the cloned fragment was confirmed by sequencing. The 2-kb fragment cassette containing the gene conferring spectinomycin resistance (I:spe′) from pH45-Ω (Fellay et al., 1989) was ligated as a Smal fragment into pGEM T-Easy digested with Smal within the ndvB gene. The resulting ndvB::Spe′ allele was cloned as a 3.3-kb NotI fragment into the sacB suicide vector pJQ200 KS digested with NotI, which was transferred afterward by triparental mating to 8004. Spectinomycin-resistant colonies were selected, and double recombinants were selected on PYM medium containing 5% sucrose and spectinomycin. The insertion in the mutant (ndvB−) was confirmed by DNA hybridization using a 32P-labeled 0.6-kb fragment from pGEM T-Easy as a probe (Figures 1A and 1B) and genomic DNA from the wild-type and ndvB− strains digested with SacI.

For the complementation experiment, the PCR product of the ndvB gene was amplified from Xcc 8004 using primers ndvB sense (5′-CCTCAATCTACAGAACTGC-3′) and ndvB antisense (5′-TGTAAG- TAGTGTCCGGGATCG-3′). The PCR fragment was cloned into pGEM T-Easy (Promega). The insert DNA was excised by digestion with EcoRI and ligated into pBRR1MC5-2 (Kovach et al., 1995). The resulting plasmid was introduced into the ndvB− strain by triparental mating.

Xcc strains were cultured in a 28°C shaker in PYM medium, and the antibiotics rifampicin (50 μg/mL), kanamycin (50 μg/mL), and spectinomycin (200 μg/mL) were used where appropriate.

Cyclic β-(1,2)-Glucan Preparation

The extracellular cyclic glucan contains 16 glucosyl residues with 15 β-1,2 linkages and 1 α-1,6 linkages. Methods to isolate the cyclic glucan from culture supernatants have been described previously (Vojnov et al., 2001). Cells of Xcc were collected by centrifugation at 10,000 g for 25 min and washed by centrifugation with 30 mM Tris-HCl buffer, pH 8. Cell pellets were extracted with 1% trichloroacetic acid to release periplasmic glucans as described by Talaga et al. (1996). The cells were then pelleted, the supernatant was neutralized by the addition of ammonium hydroxide, and the neutralized extract was concentrated by rotary evaporation. Cyclic glucans present in the culture supernatants were analyzed after precipitation of the xanthan by the addition of KCl to 1% (w/v) final concentration and 2 volumes of ethanol. The xanthan was removed by centrifugation, and the supernatant and pellet were lyophilized. The dried supernatant sample was dissolved in 5% (v/v) acetic acid prior to size-exclusion chromatography on a BioGel P4 column to separate the culture supernatant glucans.
For BioGel P4 chromatography, a column (1.5 x 42 cm) was equilibrated and eluted with 5% (v/v) acetic acid. Fractions (0.9 mL) were collected at a flow rate of 20 mL/h and assayed for carbohydrate using the anthrone reagent (Loewus, 1952). Fractions containing cyclic glucan were pooled and lyophilized. Carbohydrate analysis of the preparation was performed after hydrolysis of the sample in 1 M HCl for 4 h at 100°C. Monosaccharides were converted into alditol acetate derivatives and analyzed by gas chromatography–mass spectrometry on a G1800A GCD system (Hewlett-Packard). The only sugar detected was glucose. Partial acid hydrolysis was performed in 0.5 M HCl for 20 min at 100°C, the acid was then removed by sequential rotary evaporation with several additions of water. The partially and totally hydrolyzed samples were subjected to TLC on silica gel G in butan-1-ol:ethanol:water (5:5:4, v/v/v) with three developments. Carbohydrates were detected by spraying the plate with a solution of 5% (v/v) sulfuric acid in ethanol followed by heating at 120°C.

14C-labeled cyclic β-(1,2)-glucan was prepared by in vitro enzymatic synthesis from UDP-[14C]glucose using total membrane preparations of Xcc. Bacteria from 1-d cultures were harvested by centrifugation for 20 min, treated with lysozyme, and broken by French press treatment exactly as described by Osborn and Munson (1984). After treatment with DNase and the removal of unbroken cells by centrifugation at 10,000g for 20 min, total membranes were collected by centrifugation at 100,000g for 4 h and resuspended in 30 mM Tris-HCl, pH 8. These membrane preparations were used for in vitro biosynthesis of [14C]cyclic β-(1,2)-glucan. The standard incubation contained 6.95 μM UDP-[14C]glucose (specific activity, 287 mCi/mmol), 200 to 300 μg of protein, and 50 mM Tris-HCl, pH 8, in a total volume of 100 μL. The reaction was performed at 20°C for 1 h and stopped by boiling for 3 min, then the incubation mixture was centrifuged at 14,000g for 10 min. The supernatant was separated and was fractionated by size-exclusion chromatography on a BioGel P4 column to separate the labeled [14C]cyclic β-(1,2)-glucan product from the substrate.

Nicotiana benthamiana and Arabidopsis thaliana Inoculations

Nicotiana benthamiana plants were grown in soil in a growth chamber (DiaMed Lab Supplies) with 70% humidity and under a regime of 16 h of light (PPFD of 200 μmol photons m⁻² s⁻¹) at 23°C and 8 h of dark at 18°C. Arabidopsis thaliana ecotype Col-0 seeds were germinated on 0.8% agar. Seedlings at the two-leaf stage were then transplanted to soil and grown in a growth chamber as described above. Plants of 4 to 5 weeks old were used for the experiments.

For N. benthamiana, leaves of 4-week-old plants were inoculated by infiltration with Xcc strains (10⁷ cfu/mL in 10 mM MgCl₂) or with 10 mM MgCl₂ alone (Yun et al., 2006). For the glucan treatment experiments, leaves were preinfiltrated with either water or purified Xcc cyclic glucan in water (50 μg/mL) and then inoculated with Xcc 8004 glucan-minus mutant (ndvB⁻) or the complemented mutant (ndvB⁺) bacterium 24 h later. Inoculation was performed according to published methods (Newman et al., 1994; Yun et al., 2006). Six 0.6-cm² discs from each leaf were taken at 0, 1, 2, 3, 4, and 5 d after inoculation, and bacterial growth was monitored as described (Yun et al., 2006). For systemic susceptibility experiments, solutions of cyclic β-(1,2)-glucan (from 10 to 50 μg/mL) were infiltrated in one leaf of a 4-week-old plant, and 6 to 24 h later two upper distal leaves were infiltrated with Xcc strains. Symptoms were monitored at 5 d after inoculation, and bacterial growth was assessed as described (Yun et al., 2006).

For the experiments with radiolabeled cyclic glucan, 500 μL of a solution of 0.34 μg/mL [14C]cyclic β-(1,2)-glucan (68 pmol, 40,000 cpm) was infiltrated into N. benthamiana leaves. Leaf discs of 0.38 cm² were taken from one local and two distal leaves (up to three nodes higher) to measure the radioactivity. TLC separations of cyclic β-(1,2)-glucan and its partial and total hydrolysis products were done as described above. Glusulase activity against 14C-labeled material extracted from leaves of N. benthamiana was done as described previously (Zorreguieta et al., 1985).

For Arabidopsis inoculation, plant pots were dipped upside down for 30 s into bacterial suspensions of 10⁷ cfu/mL prepared in 10 mM MgCl₂ and 0.02% Silwet L-77. Plants were then covered with a transparent lid. Control plants were dipped in a solution of 10 mM MgCl₂ and 0.02% Silwet L-77. At the indicated time points after inoculation, leaves were removed, weighed, and then homogenized in 10 mM MgCl₂. Bacterial numbers in these homogenates were determined by plating a dilution series on PYM medium containing appropriate antibiotics. Bacterial populations were expressed as a cfu per gram fresh weight (Yun et al., 2006). For the glucan treatment experiments, plants were vacuum-infiltrated with a solution of cyclic β-(1,2)-glucan (50 μg/mL) and 24 h later dipped into bacterial suspension as described above. For the systemic susceptibility experiments, a solution of cyclic β-(1,2)-glucan (50 μg/mL) was vacuum-infiltrated in one leaf of 4-week-old plants, and 24 h later bacteria were dip-inoculated as described previously. Bacterial growth was assessed as described above.

Callose Staining

Bacteria were infiltrated into N. benthamiana plant leaves using a 1-μL syringe. Callose staining was performed at 48 h after bacterial inoculation as described by Hauck et al. (2003). Leaves were cleared of chlorophyll using alcoholic lacto-phenol and were rinsed in 50% ethanol and then in water before staining for 30 min with 0.01% aniline blue (Sigma-Aldrich) in 150 mM K₂HPO₄, pH 9.5. Samples were mounted in 50% glycerol and examined with a Zeiss Axiophot D-7082 photomicroscope with an A3 fluorescence cube. The callose depositions were quantified with Image Pro Plus software (Media Cybernetics).

RNA Gel Blot Analysis

Total RNA was isolated from N. benthamiana leaves using Tri reagent (Sigma-Aldrich). Fifteen micrograms of total RNA from each sample was separated on a 1.2% agarose gel containing formaldehyde, transferred to a Hybond-N⁺ nylon membrane, and hybridized with Nt PR-1 gene-specific probe. The filters were prehybridized and hybridized in modified Church and Gilbert buffer; 7% (w/v) SDS, 0.5 M phosphate buffer, pH 7.2, and 10 mM EDTA. Prehybridization was performed at 65°C for 2 h. Hybridization was performed overnight with the addition of a denatured 32P-labeled Nt PR-1 probe that was synthesized using the Prime-a-Gene labeling system (Promega). Membrane washing was performed according to the manufacturer’s protocol (Amersham Pharmacia Biotech). The filters were exposed to storage phosphoscreen autoradiography and screened in a Storm 820 optical scanner (Amersham Pharmacia Biotech).

Accession Number

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number XC_4168.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure 1. Xcc Cyclic β-(1,2)-Glucan Suppresses the Systemic Accumulation of Callose Deposition in N. benthamiana.

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Luciano Ariel Rigano, Caroline Payette, Geneviève Brouillard, María Rosa Marano, Laura Abramowicz, Pablo Sebastián Torres, Maximina Yun, Atilio Pedro Castagnaro, Mohamed El Oirdi, Vanessa Dufour, Florencia Malamud, John Maxwell Dow, Kamal Bouarab and Adrian Alberto Vojnov

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